Oocyte activation deficiency: a role for an oocyte contribution?

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TABLE OF CONTENTS

- Introduction
- Methods
- ART: analysis of the current data
- Activation of the oocyte is a step mediated by sperm proteins
- PLCζ: a critical SOAF
  - Phospholipases and PLCζ
  - PLCζ is present in sperm from mammalian and non-mammalian species but may differ in its mechanism of action
  - Mechanism of PLCζ action
  - PLCζ appears to be involved in functions other than oocyte activation
  - OAD occurs in sperm exhibiting PLCζ deficiencies
- Other sperm proteins as potential SOAF candidates
  - Citrate synthase and the truncated form of c-kit tyrosine kinase receptor
  - PAWP
- Controversies surrounding the relative importance of PLCζ and PAWP
- Could oocyte factors explain some cases of OAD?
- The SOAF triggers a cascade mediated by vital oocyte factors
- PIP2 and DAG
- The central role of InsP3R
  - Isoforms and regulation
  - Evidence for the role of the InsP3R during oocyte activation and maturation
  - InsP3Rs are present in oocytes from mammalian and non-mammalian species, but their intracellular distribution can differ between species
- PKC
  - The PKC family
  - PKC and the mammalian oocyte
  - The role of PKCa during oocyte activation
  - PKCs, oocyte maturation and early embryo development
- Ca2+ oscillations and homeostasis in the oocyte
  - Membrane channels and SOCE: STIM1 and ORAI1
  - Sarco/endoplasmic reticulum and plasma membrane Ca2+-ATPases
- Other oocyte proteins that could underlie oocyte activation failure
  - CaMKII and MAPK
  - Other phospholipases
  - CDK1 and CHERP
Introduction

Infertility is defined as the inability of a sexually active couple to achieve pregnancy after 1 year of unprotected vaginal intercourse (Practice Committee of the American Society for Reproductive Medicine, 2013) and is considered as a disease by the World Health Organization (Zegers-Hochschild et al., 2009). Infertility affects approximately one in seven couples in the UK (National Collaborating Centre for Women’s and Children’s Health, UK, 2013) and between 10 and 16% of couples world-wide (Fidler and Bernstein, 1999; Boivin et al., 2002; Louis et al., 2013; Thoma et al., 2013; Chandra et al., 2014; ESHRE, ART Fact Sheet, June, 2014). Infertility figures differ between primary and secondary causes, and amongst developed, developing and underdeveloped countries. When considering the population worldwide, primary infertility affects between 1 and 8% of couples, while secondary infertility, defined as the inability of a woman to become pregnant or to carry a pregnancy to term after the birth of one or more children, may occur in up to 35% of cases in developing countries (Vayena et al., 2002).

The absence of pregnancy, or the experience of miscarriage, accounts for many other consequences, including psychological distress (Bak et al., 2012), economic constraints (Wu et al., 2013) and social stigmatization (Slade et al., 2007). Furthermore, infertility and sperm quality have been suggested as being markers of overall male health in addition to lifestyle and/or social factors, and men presenting a low semen volume, low percentage of motile and morphologically normal sperm appear to have higher mortality odds (Jensen et al., 2009).

Current data indicate that 20–30% of infertile cases are predominantly related to a male factor, 20–35% of cases to a female factor, and 25–40% of cases to problems associated with both male and female factors. Moreover, the precise cause of infertility remains unexplained in around 10–25% cases. Although figures can differ between studies, general consensus indicates that infertility is more likely to be related to a female factor, or male and female factors collectively, than a male only cause (Thonneau et al., 1991; De Kretser, 1997; Anderson et al., 2009; National Collaborating Centre for Women’s and Children’s Health, UK, 2013; ESHRE, ART Fact Sheet, 2014).
In the case of males, semen and sperm defects are a major cause of infertility including deficient spermatogenesis and/or abnormal epididymal maturation that may have a genetic basis (Matzuk and Lamb, 2008). These deficiencies are related not only to the basic cellular functions required for mitosis, meiosis, sperm differentiation, and sperm chromatin integrity, but also to vital proteins that are of paramount importance for the events that take place following sperm–oocyte fusion (Robinson et al., 2012; López et al., 2013; Pregl Breznik et al., 2013; Simon et al., 2013).

In women, abnormalities underlying infertility primarily involve ovarian and ovulatory disorders such as primary ovarian insufficiency and polycystic ovarian syndrome (25%), tubal damage (20%), and uterine or peri- toneal disorders (10%) (Data given here relate to the total percentage of infertile cases). Endometriosis, pelvic conditions, embryo defects, previous infections from Chlamydia trachomatis and susceptibility to rubella may also influence the ability to conceive (National Collaborating Centre for Women’s and Children’s Health, (UK), 2013). Despite the identification of genetic causes in some cases of infertility, further advancement in their diagnosis and treatment is required. Indeed, disorders such as primary ovarian insufficiency premature and primary ovarian failure (independent of hypothalamic and pituitary defects) have yet to be determined (reviewed in Matzuk and Lamb, 2008).

Since factors that may underlie infertility are of a very broad nature, advice for treatment requires correct diagnosis of the specific cause. Currently, three different types of treatment are available clinically: surgical, for example laparoscopy for ablation of endometriosis; medical, for example the use of drugs to induce ovulation; assisted reproductive technology (ART), which comprises any treatment in which conception is performed by means other than vaginal coitus. IVF, ICSI and subsequent embryo transfer are the main ART procedures, and all involve handling of gametes or embryos in a laboratory environment. Many cases of infertility can now be rectified by ART, such as IVF (e.g. in cases of female tubal damage, ovulatory failure; Stern, 2012) and ICSI (in male-factor infertility for conditions such as oligozoospermia; Kashir et al., 2010).

As this review focuses upon the factors that may lead to oocyte activation deficiency (OAD) and subsequent fertilization failure, the systemic analysis of published data arising from ART can shed significant light upon the factors that may be involved in the failure of these techniques and their relative association with OAD.

**Methods**

This work is based upon information gathered from a critical review of the literature arising from PubMed, performed between April 2014 and July 2015, and targeting studies concerning sperm and oocyte factors that could account for OAD. This search included studies of in vitro oocyte maturation in human oocytes and work arising from animal models. In addition, we focused upon clinical data available for sperm- and oocyte-related OAD. We also include a section about artificial oocyte activation (AOA), which summarizes recent discussion about this technique, and the concerns associated with its clinical use.

**ART: analysis of the current data**

Since the birth of the first in vitro baby in 1978, ART has emerged as a powerful tool for those couples seeking treatment for infertility (Johnson, 2011). Globally, around 1.5 million ART cycles are performed per year, with an estimated 350,000 babies born worldwide. Figures released by the European Society for Human Reproduction and Embryology (ESHRE) show that pregnancy and delivery rates following IVF and ICSI barely exceed 32 and 33%, respectively (De Mouzon et al., 2009; Kupka et al., 2014). According to Mascarenhas et al. (2012), pregnancy rates per embryo transfer are 23.4% after frozen embryo transfer and 47.5% after oocyte donation.

To date, IVF and ICSI have been proven to be very successful, and 1.5% of all births in the UK now result from the use of ART (reviewed in Ramadan et al., 2012; HFEA, 2014). However, while the use of IVF has been reported to be a suitable treatment for many infertile couples, normal IVF cycles can still fail in some cases (Bhattacharya et al., 2013). In this scenario, ICSI, which consists of the direct injection of a single spermatozoon into the oocyte (Palermo et al., 1992), is advised and has proven to be very effective with a mean fertilization rate of ~80% (Vanden Meerschaut et al., 2013, 2014a; Neri et al., 2014). According to the latest figures released by the Human Fertilisation and Embryology Authority (HFEA), ~52% of ART cycles in the UK now involve ICSI (HFEA, 2014).

Figure 1 shows the main causes for total fertilization failure (TFF) following IVF and ICSI treatments, using data obtained by the HFEA for 2012 (HFEA, 2014). While male factors account for 10.0% of failed IVF cases and for 50.4% of failed ICSI cases, unexplained and uncategories causes have been attributed to 50.3% (IVF) and 25.8% (ICSI) of TFF cases. The differences and similarities between IVF and ICSI may help us to understand what underlies the unexplained/unategorized causes in both cases. In IVF, capacitated sperm have to interact with the oocyte zona pellucida, the acrosome reaction needs to be triggered, and further fusion between sperm and oocyte membranes needs to occur (reviewed in Barroso et al., 2009). However, in the case of ICSI, there is no direct interaction between sperm and oocyte membranes, but rather the oocyte membrane is mechanically pierced and biological obstacles are surpassed (Palermo et al., 1992; Beck-Fruchter et al., 2014). Therefore, IVF failure can be associated with asthenozoospermia, inability of sperm to capacitate properly, failure to undergo acrosome release, or problems during gamete fusion, given the crucial interaction between Juno-Izumo and the role of lipid raft domains in the oocyte membrane (Van Blerkom and Caltrider, 2013; Bianchi et al., 2014). In contrast, the main cause of ICSI failure is related to a male factor in ~50% of cases (Pregl Breznik et al., 2013; Zhao et al., 2014a).

Although ICSI is a procedure that allows any form of sperm to fertilize an oocyte and thus rescue fertility in some cases, complete fertilization failure still occurs in 1–5% of ICSI cycles (Mangoli et al., 2008; Yanagida et al., 2008; Nasr-ESfahani et al., 2010; Vanden Meerschaut et al., 2013, 2014a), which in the case of the UK affects at least 1200 couples annually (Amdani et al., 2013). ICSI failure can be attributed to several factors, such as technical factors (e.g. incorrect injection or sperm expulsion during injection), sperm defects and biochemical factors (e.g. poor sperm nuclear chromatin condensation, oocyte spindle defects) (Swain and Pool, 2008; Yanagida et al., 2008). However, OAD is generally regarded as the principal cause of fertilization failure following ICSI, accounting for an estimated 40% of failed cases (Flaherty et al., 1998; Rawe et al., 2000; Mahutte and Arici, 2003; Vanden Meerschaut et al., 2013, 2014a). In this regard, it is worth mentioning that the causes of OAD are not necessarily restricted to sperm defects but may also involve the oocyte, since oocyte quality also plays a pivotal role in fertilization success (Kashir et al., 2010; Ramadan et al., 2012; Neri et al., 2014).
Therefore, the present work first considers recent oocyte activation studies which have focused upon sperm factors, and in particular, the role of phospholipase C zeta ($\text{PLC}_\zeta$). After reviewing the crucial role of this protein for OAD and its clinical significance, we discuss whether oocyte factors might also have a role to play. While this is a very relevant and pertinent issue, it has received far less attention in the literature, and may open up several options for future research.

**Activation of the oocyte is a step mediated by sperm proteins**

It is well understood that mature oocytes remain arrested at metaphase-II (M-II) when ovulated (Stricker, 1999; Kashir et al., 2013a) and are only released from this state when they are fertilized by a spermatozoon in a process known as ‘oocyte activation’ (Jones, 2007; Machaca, 2007; Horner and Wollner, 2008; Dale et al., 2010). The presence of a sperm in the cytoplasm of a mammalian oocyte evokes a characteristic pattern of intracellular calcium ($\text{Ca}^{2+}$) oscillations (Swann, 1990; Kline and Kline, 1992a) that orchestrate a series of further key events, such as cortical granule exocytosis, prevention of polyspermy, polar body extrusion, cytoskeletal rearrangements, resumption of meiosis, formation of pronuclei, initiation of the first mitotic division in the new zygote, recruitment of maternal mRNA, and regulation of gene expression (Abassi and Foltz, 1994; Swann et al., 2004; Swann and Yu, 2008; Kashir et al., 2014; Fig. 2). The release of these periodic $\text{Ca}^{2+}$ oscillations over a specific temporal window is critical not only for oocyte activation, but also for the initiation of embryogenesis (Ramadan et al., 2012; Amdani et al., 2013).

The precise mechanism by which fertilized oocytes are released from M-II arrest was the source of much debate that dates back decades. Three theories were proposed to explain this event: The ‘sperm factor theory’ which suggested that a catalytic substance in the sperm head initiated $\text{Ca}^{2+}$ release in the ooplasm following gamete fusion (Dale et al., 1985; Fissore et al., 1999a); The ‘receptor theory’ in which a signal transduction pathway was proposed to be triggered after a receptor located on the oolemma interacted with a sperm ligand (Kline et al., 1988; reviewed in Parrington et al., 2007); The ‘calcium bomb’ hypothesis in which $\text{Ca}^{2+}$ would enter the oocyte upon fertilization either from stores in the sperm or through channels on the sperm plasma membrane (Jaffe, 1983). The ‘calcium bomb’ theory is now largely rejected, as too is ‘the receptor theory’ (Williams et al., 1998). While the receptor theory gained some credence initially, a sperm ligand/oocyte receptor mechanism that can induce the release of $\text{Ca}^{2+}$ in a mammalian oocyte has yet to be identified and ICSI can successfully initiate activation bypassing any potential membrane mechanism completely (Neri et al., 2014).

New technologies such as calcium imaging have allowed significant steps forward in the last three decades (Roy and Hajnóczky, 2008; Swann et al., 2009; Bruton et al., 2012; Swann, 2013), with growing evidence for the ‘sperm factor theory’. Strikingly, sperm soluble extracts directly injected into the ooplasm were seen to induce $\text{Ca}^{2+}$ oscillations (Jones et al., 1998; Parrington et al., 1998a), and the microinjection of hamster and boar sperm cytoplasmic extracts into hamster oocytes were also able to trigger $\text{Ca}^{2+}$ oscillations similar to those observed during IVF of hamster oocytes (Swann, 1990).

Further knowledge concerning the sperm activation mechanism in non-mammalian eggs was gained during the 1990s and the first decade of the 21st century (Swann and Whitaker, 1990). The first protein proposed as a sperm-borne oocyte activation factor (SOAF) in mammals was oscillin, this nomenclature being derived from the $\text{Ca}^{2+}$ oscillations believed to be evoked by this protein following
fertilization (Parrington et al., 1996; Montag et al., 1998). However, later reports using a recombinant oscillin could not confirm this role, and additional evidence indicated that a homologue of oscillin (known as human glucosamine-6-phosphate isomerise) was unable to induce \( \text{Ca}^{2+} \) oscillations upon injection into mouse oocytes (Tesarik, 1998; Fissore et al., 1999a; Wolny et al., 1999). Accumulating biochemical and clinical evidence now strongly indicates that \( \text{PLC}\zeta \) (1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase zeta-1; accession number: Q86YW0) is the key SOAF that activates the oocyte upon gamete fusion (Saunders et al., 2002; Kashir et al., 2010; Amdani et al., 2013). However, it is important to note that three other sperm proteins (citrate synthase, truncated c-Kit tyrosine kinase and post-acrosomal WWP-domain binding protein, PAWP) have either been put forward as being SOAF candidates, or have been reported to be involved in the oocyte activation mechanism (Sette et al., 2002; Harada et al., 2007; Wu et al., 2007a, b).

**Figure 2** Oocyte activation triggered by a sperm-specific factor, phospholipase C zeta (\( \text{PLC}\zeta \)). Upon oocyte-sperm membrane fusion, \( \text{PLC}\zeta \) is released into the ooplasm and interacts with an as-yet unknown oocyte-borne factor (or factors), thereby facilitating the hydrolysis of PIP\(_2\) into DAG and InsP\(_3\). InsP\(_3\) subsequently triggers \( \text{Ca}^{2+} \) release from intracellular stores and thus initiates a cascade of events that finally alleviates MII-arrest. Briefly, \( \text{Ca}^{2+} \) oscillations mediate the release of cortical granules, deactivate MAPK which allows the formation of pronuclei, and activates CaMKII. Activation of CaMKII in turn inhibits CSF (Emi2) which then liberates APC. APC reduced the levels of Cyclin B1 in the maturation-promoting factor (MPF) complex comprising CDK1 and Cyclin B1. Degradation of Cyclin B1 inactivates the MPF which, in turn, releases the oocyte from MII-arrest. APC: anaphase-promoting complex/cyclosome; CaM/CalMM: calcium/calmodulin-dependent protein kinase II; CSF: cytostatic factor; CNB1: cyclin B1; CDK1: cyclin-dependent kinase 1; DAG: diacylglycerol; InsP\(_3\): inositol 1,4,5-trisphosphate; InsP\(_3\)R: InsP\(_3\) receptor; MAPK: mitogen-activated protein kinase; PIP\(_2\): phosphatidylinositol 4,5-bisphosphate; PKC: protein kinase C.

**PLC\zeta: a critical SOAF**

**Phospholipases and PLC\zeta**

PLC enzymes participate in one of the most common pathways in intracellular signal transduction (Kadamur and Ross, 2013; Rhee, 2013). In this general mechanism, PLCs hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) from plasma membrane sources into inositol 1,4,5-trisphosphate (InsP\(_3\)) and diacylglycerol (DAG) (Yang et al., 2013). Inositol 1,4,5-trisphosphate then interacts with its receptor (InsP\(_3\)R) found on the membrane of the endoplasmic reticulum (ER), which in turn allows \( \text{Ca}^{2+} \) release from internal ER stores. DAG, which remains attached to the plasma membrane, together with the \( \text{Ca}^{2+} \) released from ER, then activates protein kinase C (PKC) (Fukami et al., 2010; Antal and Newton, 2014; Taylor et al., 2014).

Currently, evidence is rapidly accumulating to indicate that \( \text{PLC}\zeta \) is the key SOAF responsible for the activation of oocytes (Wu et al., 2001; Cox...
PLCz is present in sperm from mammalian and non-mammalian species but may differ in its mechanism of action

PLCz has not only been identified in human sperm but also in other mammalian species, including hamster, pig, monkey and horse (Cox et al., 2002; Saunders et al., 2007; Yoneda et al., 2002; Nomikos et al., 2013a). In fertile men, PLCz is found in the equatorial and post-acrosomal regions (Fujimoto et al., 2004; Yoon et al., 2008), and observations using transmission electron microscopy following immunogold staining have suggested that this protein is located in the perinuclear theca and along the inner acrosomal membrane (Escoffier et al., 2015). Upon capacitation, and even following the acrosome reaction, PLCz remains localized at the post-acrosomal region (Grasa et al., 2008). This pattern of localization supports the role of PLCz as the SOAF, since after the acrosome reaction, the equatorial region of the sperm membrane is exposed, adheres to and fuses with the oolemma (Barroso et al., 2009). In strong support of the role of PLCz, is the fact that mammalian oocytes can be activated following microinjection of either recombinant PLCz or complementary RNA (cRNA) encoding PLCz (Yoon et al., 2008; Swann et al., 2012; Nomikos et al., 2013b).

Mechanism of PLCz action

To date, the exact process by which PLCz activates the molecular mechanisms that promote oocyte activation still remains unclear, although the critical roles played by its discrete functional domains are highly apparent (Nomikos et al., 2005). In this regard, one has to consider that PLCz is the smallest PLC isoform in mammals with a molecular mass of 70.4 kDa in the case of humans (Nomikos et al., 2013a). PLCz comprises four EF hand domains, a C2 domain, and catalytic X and Y core domains. Kouchi et al. (2005) reported that EF1 and EF2 domains are important for protein activity, while EF3 is responsible for its high Ca2+ sensitivity (Yoda et al., 2004). Screening interactions between the C2 domain and phosphoinositides revealed that the C2 domain exhibits substantial affinity for phosphatidylinositol 3-phosphate and for phosphatidylinositol 5-phosphate but not for PIP2, and that phosphatidylinositol 3-phosphate and phosphatidylinositol 5-phosphate reduce PLCz activity via a negative-regulatory mechanism (Kouchi et al., 2005).

Most PLC isoforms (e.g. PLCδ1), but not PLCz, reside in the plasma membrane, are linked to G protein-coupled receptors and possess a PH domain (Williams et al., 1998; White et al., 2010). While the PH domain typically binds to and hydrolyses PIP2 found in the plasma membrane (Suh et al., 2008; Bunney and Katan, 2011), PLCz is devoid of this particular domain and instead, targets its substrate via the X-Y linker region and PIP2-containing vesicles distributed across the ooplasm (Yu et al., 2012; Theodoridou et al., 2013). Therefore, upon the fusion of sperm and oocyte plasma membranes, PLCz has been proposed to diffuse across the ooplasm within the space of 10 min, target PIP2 located in oocyte vesicles and initiate the first calcium wave within 30 min of gamete fusion (Saunders et al., 2007; Swann and Lai, 2013).

Finally, and aside from the absence of a PH domain, there is another key feature that makes PLCz different from the other somatic isoforms (-β, -γ, -δ and -ε), since the XY-linker region of PLCz does not mediate catalytic auto-inhibition, but rather confers maximal enzymatic activity (Nomikos et al., 2007, 2011b; Yu et al., 2012).

PLCz appears to be involved in functions other than oocyte activation

While it is clear that PLCz is involved in oocyte activation, its involvement in other vital reproductive events has also been suggested (Young et al., 2009). For example, there is emerging evidence for a potential role of PLCz during early embryonic division. In mouse, for example, Ca2+-oscillations occur when PLCz is distributed throughout the ooplasm and cease when the nuclear localization signal drives PLCz into the developing pronuclei (Larman et al., 2004). Once the first mitotic division is initiated, Ca2+-oscillations are resumed, presumably because PLCz is released into the ooplasm following the breakdown of nuclear envelope (Larman et al., 2004; Yoda et al., 2004). Therefore, while the function of these mitotic Ca2+-spikes remains to be elucidated, it appears that they exert an effect upon both pre- and post-implantation development in a manner that is independent of their ability to activate oocytes (Sone et al., 2005). In this context, it is worth mentioning that not only are the oocytes able to decipher a specific pattern of Ca2+-oscillations, but also gene expression profiles in the early embryo rely on this signalling pathway (Ozil et al., 2006).
OAD occurs in sperm exhibiting PLCζ deficiencies

Defective fertilizing sperm are known to cause arrest of development at multiple levels during embryo preimplantation development (Colombo et al., 1999; Barroso et al., 2009). Thus, one of the most relevant aspects that merits particular attention is the relationship between PLCζ and male infertility. Whilst fertile men present, overall, a significantly higher proportion of sperm exhibiting PLCζ than infertile men (Kashir et al., 2013b; Yelumalai et al., 2015), deficiencies, abnormal localization, reduced activity/expression, or genetic mutations in PLCζ have been associated with OAD and ICSI failure (Yoon et al., 2008; Heytens et al., 2009, 2010; Aghajanpour et al., 2011; Kashir et al., 2011, 2012a, c; Nomikos et al., 2011a; Lee et al., 2014; Yelumalai et al., 2015). The presence of morphologically abnormal spermatozoa has also been related to deficient forms of PLCζ, and the total absence of PLCζ (Yoon et al., 2008; Heytens et al., 2009; Lee et al., 2014). One of the most severe and rare cases (incidence <0.1%) is globozoospermia (see Dam et al., 2007) in which morphologically abnormal sperm without acrosomes and significant DNA fragmentation can be totally devoid of PLCζ (Taylor et al., 2010; Kashir et al., 2012b; Yassine et al., 2015).

Furthermore, two point mutations in the PLCζ gene have been identified in a non-globozoospermic infertile patient. These mutations caused the substitution of histidine to proline at residue 398 (H398P) within the Y domain, and the substitution of histidine to leucine (H233L) within the X domain, resulting in a significantly impaired ability to activate the oocyte (Heytens et al., 2009; Kashir et al., 2012a, c).

Other sperm proteins as potential SOAF candidates

To date, four sperm-borne proteins have been put forward as predominant candidates for the SOAF. These four proteins are PLCζ, a truncated form of c-kit tyrosine kinase receptor, citrate synthase, and PAWP. While there is extensive evidence for the role of PLCζ (reviewed in previous section), the relative importance of the other three proteins is far less clear, and in the case of PAWP, rather controversial at present.

Citrate synthase and the truncated form of c-kit tyrosine kinase receptor

The truncated form of c-kit tyrosine kinase receptor (tr-kit) is present in the equatorial and post-acrosomal sheath of sperm (Muciaccia et al., 2010), and has been reported to alleviate M-II arrest in mouse oocytes and trigger other events of early embryogenesis such as cortical granule exocytosis and the formation of pronuclei (Sette et al., 1997, 2002; Rossi et al., 2003). According to Sette et al. (2002), tr-kit might interact with Fyn, a Src-like tyrosine kinase residing in the oocyte cortex, and Fyn (or perhaps another kinase) would then phosphorylate a tyrosine residue of tr-kit (Tyry16). This would allow tr-kit to interact with the SH2 domain of Fyn and subsequently permit the phosphorylation of a tyrosine residue (Tyry81) in the SH region (Carpenter and Ji, 1999). The tr-kit/Fyn complex would thus finally activate the oocyte by interacting with the SH3 domain of PLCγ1 (Sette et al., 1998, 2002). However, while the role of PLCγ has been investigated in non-mammalian species (Sato et al., 2006), tr-kit has yet to be proven to elicit Ca2+ oscillations in oocytes. This makes it difficult to consider tr-kit further as a SOAF in mammals.

On the other hand, the suggested role for citrate synthase arose from the work of Harada et al. (2007) in amphibians. These authors observed that the protein responsible for egg activation in the newt Cynops pyrhogaster was 45 KDa and resided in the neck and intermediate piece of the sperm. This protein was partly homologous to citrate synthase in Xenopus. However, injection of mouse PLCζ was also able to activate newt eggs. Although this may not explain what happens in mammals, it does highlight the variety of mechanisms present in the vertebrate kingdom. In fact, general consensus of opinion amongst researchers has now discarded tr-kit and citrate synthase as SOAF candidates in mammals, with most evidence supporting the role of PLCζ and emerging data indicating a potential role for PAWP.

PAWP

PAWP (also known as WB2P) is a sperm protein exclusively located in the post-acrosomal sheath of the sperm perinuclear theca (Wu et al., 2007a, b), which surrounds the nucleus except in the caudal region where the tail is implanted. This region consists of a condensed protein layer that represents the major cytosolic fraction in the sperm head and is made up of two different regions: the sub-acrosomal layer and the post-acrosomal sheath (Oko and Sutovsky, 2009; Ferrer et al., 2012). Given the dynamics of sperm interaction with the oocyte, the post-acrosomal sheath has been suggested to be involved in oocyte activation (Sutovsky et al., 2003; Toshimori and Ito, 2003; Ito et al., 2009, 2010).

While some authors have found that injecting recombinant PAWP or protein extracts from the perinuclear theca into porcine, bovine, macaque, human, and Xenopus oocytes alleviates M-II arrest (Wu et al., 2007a; Aarabi et al., 2010, 2014a; Kennedy et al., 2014), others report that PAWP does not trigger calcium oscillations in mouse oocytes (Nomikos et al., 2014, 2015a). Furthermore, total levels of PAWP evaluated through immunohistochemistry have been associated with sperm quality and fertilizing ability in bulls (Kennedy et al., 2014), and with fertilization rates and embryo development following ICSI in humans (Aarabi et al., 2014b).

Whether PAWP acts as a SOAF remains to be fully confirmed, but in the context of the present review, evidence does appear to indicate a possible role in oocyte activation. If this was the case, failures in some of the oocyte proteins interacting with PAWP could also underlie some cases of OAD. The C-terminal PAWP region is rich in proline and presents a functional PPXY consensus sequence that binds WW-group I protein domains. These WW domains mediate protein–protein interactions in a way that is similar to SH3 domains. When the PPXY region is blocked, PAWP does not activate the oocyte. For this reason, Aarabi et al. (2014a) suggested that the PPXY domain present in PAWP interacts with the WW-group I protein domain from PLCγ following a non-canonical pathway. The activation of PLCγ would then hydrolyse PIP2 and this would initiate Ca2+ oscillations. This mechanism, however, requires further research in order to validate its authenticity and any relevance to oocyte activation. Such additional work is particularly important because other reviews have pointed out that PLCγ does not mediate Ca2+ oscillations in mouse oocytes, and also that the negative-control peptide used by Aarabi et al. (2014a) did...
not affect the pattern of sperm-induced Ca\(^{2+}\) oscillations (Nomikos et al., 2015b).

### Controversies surrounding the relative importance of PL\(C\)\(\zeta\) and PAWP

Although PL\(C\)\(\zeta\) has been accepted as the SOAF for over a decade, the authors that first proposed PAWP as the SOAF also raised concerns about PL\(C\)\(\zeta\), largely due to the fact that PL\(C\)\(\zeta\) is found in mouse and human sperm compartments other than the perinuclear theca where SOAF should be expected to reside (Aarabi et al., 2012). In addition, Aarabi et al. (2012) challenged the role of PL\(C\)\(\zeta\) as they failed to detect PL\(C\)\(\zeta\) in spermatids at the end of spermiogenesis, and failed to demonstrate PL\(C\)\(\zeta\)-immunostaining following the acrosome reaction, zona pellicuda penetration, sperm–oocyte fusion, and sperm entry into the ooplasm. Thus, in contrast to Escoffier et al. (2015), Aarabi et al. (2012) did not find PL\(C\)\(\zeta\) in the perinuclear theca of mouse and human sperm.

Furthermore, Aarabi et al. (2012) also suggested that principal epididymal cells secrete PL\(C\)\(\zeta\). With regard to this, it is worth mentioning that epididymal maturation involves the rearrangement of a variety of proteins, mainly on the sperm membrane surface (Dacheux and Dacheux, 2013). However, PL\(C\)\(\zeta\) has been identified in the perinuclear theca (Escoffier et al., 2015) and in the soluble-chromatin fraction of the human sperm head (Castillo et al., 2014). If PL\(C\)\(\zeta\) is in the sperm chromatin soluble fraction, i.e. proteins that are weakly attached to the sperm DNA and play a regulatory rather than structural role, it is quite unlikely that it arises from epididymal maturation, but rather it should arise during spermatogenesis.

Finally, while Aarabi et al. (2014b) demonstrated a link between PAWP and fertility outcomes from ICSI, reduced levels of PL\(C\)\(\zeta\) (Kashir et al., 2013b) or genetic mutations in the PL\(C\)\(\zeta\) coding region (Heytens et al., 2009; Kashir et al., 2012a) also affect fertility outcomes and can be rescued with PL\(C\)\(\zeta\) cRNA and recombinant protein (Yoon et al., 2008; Nomikos et al., 2013b; Yelumalai et al., 2015). Consequently, it is not yet clear whether there is one sole oocyte activation factor or more, or whether different factors work together (Table I). In any case, this issue remains the source of significant debate at present, because other research groups have failed to repeat the experiments providing evidence for PAWP (Nomikos et al., 2014, 2015b; Aarabi et al., 2015; Amdani et al., 2015; Vadnais and Gerton, 2015).

### Could oocyte factors explain some cases of OAD?

In a recent study, total levels and proportions of sperm exhibiting PL\(C\)\(\zeta\) were found to be significantly correlated with ICSI fertilization outcomes; however, not all cases of TFF were explained by deficiencies in this protein (Yelumalai et al., 2015), at least using the tools and techniques deployed in this particular study, which relied heavily upon the use of a polyclonal antibody. While the specificity of the antibody used by Yelumalai et al. (2015) has been confirmed in this and other studies (Grasa et al., 2008; Kashir et al., 2013b), and supported by appropriate peptide blocking assays, it should, of course, be noted that the disparate specificity of polyclonal antibodies could influence experimental outcomes (Ramadan et al., 2012; Amdani et al., 2013, 2015; Kashir et al., 2013b; Nomikos et al., 2013b). The entire body of work described thus far in the literature for PL\(C\)\(\zeta\) localization, by ourselves and other research groups, relies solely upon the use of polyclonal antibodies. It is therefore prudent to treat such data with a certain amount of caution until such work has been verified by a more powerful and reliable generation of monoclonal antibodies.

However, aside from this concern, and while it could be argued that sperm factors other than PL\(C\)\(\zeta\) could also account for OAD, it is entirely reasonable to venture that oocyte factors may also contribute to such failure since the series of critical downstream signalling events triggered by the sperm rely heavily upon oocyte-borne factors. This is supported

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**Table I  PL\(C\)\(\zeta\), PAWP, and implications for oocyte activation deficiency (OAD).**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Description</th>
<th>Possible contribution to OAD</th>
<th>References</th>
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<tr>
<td>PL(C)(\zeta)</td>
<td>Significant evidence already exists for PL(C)(\zeta)’s primary role as the SOAF. PL(C)(\zeta) hydrolyses PIP(_2) into InsP(_3) and DAG and elicits Ca(^{2+}) oscillations within the oocyte. In fertile men, PL(C)(\zeta) is located along the inner acrosomal membrane, and in the perinuclear theca of the equatorial and post-acrosomal regions.</td>
<td>Fertile men present a significantly higher proportion of sperm exhibiting PL(C)(\zeta) than infertile men. Deficiencies, abnormal localization, activity/expression, or genetic mutations in PL(C)(\zeta) have been linked to OAD and ICSI failure. Two point mutations in the PL(C)(\zeta) gene have been identified in a non-globozoospermic infertile patient resulting in a significantly impaired ability to activate oocytes.</td>
<td>Cox et al. (2002); Escoffier et al. (2015); Fujimoto et al. (2004); Heytens et al. (2009); Heytens et al. (2010); Kashir et al. (2012a); Kashir et al. (2012c); Kouchi et al. (2004); Lee et al. (2014); Nomikos et al. (2011a); Saunders et al. (2002)</td>
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<td>PAWP</td>
<td>PAWP is another sperm-specific protein that has been recently proposed as a SOAF. This protein is exclusively located in the post-acrosomal sheath of the sperm perinuclear theca. Exact relevance has yet to be confirmed but could play a role in oocyte activation, whether a SOAF role is proven or not.</td>
<td>Total levels of PAWP evaluated via immunohistochemistry have been associated with sperm quality and fertilizing ability in bulls, and with fertilization rates and embryo development following ICSI in humans.</td>
<td>Aarabi et al. (2010); Aarabi et al. (2014a); Aarabi et al. (2014b); Kennedy et al. (2014); Wu et al. (2007a); Wu et al. (2007b)</td>
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PL\(C\)\(\zeta\): phospholipase \(\zeta\) zeta; PAWP: post-acrosomal WWP-domain binding protein; SOAF: sperm-borne oocyte activation factor; DAG: diacylglycerol; InsP\(_3\): inositol 1,4,5-trisphosphate.
Oocyte factors and activation deficiency

The SOAF triggers a cascade mediated by vital oocyte factors

It is worth noting that although different sperm proteins have been proposed to be the SOAF, all candidates have been suggested to evoke Ca\(^{2+}\) oscillations and are known to be involved in a PLC/phosphoinositide transduction pathway (Wu et al., 2001). So, either the SOAF itself is a PLC, as in the case of PLC\(\xi\) (Saunders et al., 2002; Kashir et al., 2010; Swann and Lai, 2013), or it involves the activation of oocyte PLCs, as suggested for PAWP (Aarabi et al., 2014a). In any case, both PLC\(\xi\) and PAWP trigger a downstream cascade that requires oocyte factors. Additionally, there are other signs indicating that the molecules that interact with the SOAF are oocyte-specific. Indeed, while PLC\(\xi\) can be expressed in somatic cells, such as those from the Chinese hamster ovary, it is unable to elicit Ca\(^{2+}\) oscillations in these cells (Phillips et al., 2011). This suggests that either one or more unknown specific proteins that are only present in the oocyte interact with PLC\(\xi\) resulting in Ca\(^{2+}\) oscillations, or that PLC\(\xi\) activity is inhibited by somatic cell factors.

On the other hand, specific peculiarities of PLC\(\xi\) render this phospholipase different from others, which in turn suggests a particular mechanism of action that has yet to be fully elucidated. As mentioned earlier, PLC\(\xi\) is the only PLC that does not possess a PH domain (Saunders et al., 2002). In other phospholipases, the PH domain targets PI(4,5)P\(_2\) located on the inner leaflet of the plasma membrane (Garca et al., 1995; Tuzi et al., 2003; Suh et al., 2008; Bunney and Katan, 2011). Instead, PLC\(\xi\) appears to hydrolyse PI(3,4,5)P\(_3\) molecules that are present in oocyte vesicles distributed across the cytoplasm rather than those present in the inner oolemma leaflet (Yu et al., 2012). This not only matches previous observations made by Yu et al. (2008), who reported that DAG generated by PLC\(\xi\) is not found in the oolemma, but also raises further questions such as whether the targeting of PI(3,4,5)P\(_3\) residing in oocyte vesicles is mediated by one or more than one oocyte-specific protein/s located in the membrane of those vesicles, as hypothesized recently (Swann and Lai, 2013). Although this theory has yet to be confirmed, it is entirely reasonable, and would explain the specificity of PLC\(\xi\) for by outcomes from the mouse oocyte activation test (MOAT; Hendryckx et al., 2005) since in some couples that fail ICSI the male partner presents a sufficient number of sperm exhibiting PLC\(\xi\) and the MOAT shows that such sperm are fully intact and functional. While it should be noted that the results of Ca\(^{2+}\) imaging in mouse oocytes cannot be directly extrapolated to human oocytes because human PLC\(\xi\) presents higher potency than the mouse isoform, it is assumed that MOAT-activation deficiency might be due to an oocyte-borne factor (Hendryckx et al., 2005, 2008). In any case, reviewing the potential contribution of the oocyte to OAD appears highly prudent at this time.

Clinical data that specifically discuss the possibility of oocyte-related OAD remain scarce. In this regard, one needs to take into account that while sperm causes may be evaluated via the assessment of PLC\(\xi\) or by the MOAT, ethical issues and restrictions on the availability of oocytes for research render it much more difficult to evaluate oocyte factors. In contrast, Vanden Meerschaut et al. (2012) reported cases of oocyte-related OAD that could not be rescued by AOA, but which was explained by the presence of multiple polar bodies and two disorganized spindle structures. In contrast, Vanden Meerschaut et al. (2012) reported cases of oocyte-related OAD that could be overcome by AOA. This led these authors to suggest that AOA could be only effective when an underlying cytoplasmic defect, rather than a structural cytoskeletal-related or nuclear deficiency, was the cause of the oocyte-borne OAD. Finally, a retrospective study found that 75% of a cohort of patients with TFF following ICSI was due to a male factor while 25% was attributed to an explained infertility (Shinar et al., 2014). It is worth noting that in their analysis, these authors did not include cases of poor ovarian reserve related to advanced age, and included cycles in which five or more mature MII-oocytes were retrieved. Using these data, these authors specifically highlighted the relevance of the oocyte in cases of TFF, since ICSI with the SOAF triggers a cascade mediated by vital oocyte factors. Addi- tionally, there are other signs indicating that the molecules that interact with the SOAF are oocyte-specific. Indeed, while PLC\(\xi\) can be expressed in somatic cells, such as those from the Chinese hamster ovary, it is unable to elicit Ca\(^{2+}\) oscillations in these cells (Phillips et al., 2011). This suggests that either one or more unknown specific proteins that are only present in the oocyte interact with PLC\(\xi\) resulting in Ca\(^{2+}\) oscillations, or that PLC\(\xi\) activity is inhibited by somatic cell factors.

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Table II Potential oocyte factors with implications for OAD (I): PIP2, InsP3 receptor and protein kinase C.

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<tr>
<td>PIP2</td>
<td>PLCζ specifically targets PIP2 found in the membrane of intracellular oocyte vesicles. PIP2 is then hydrolysed into InsP3 and DAG.</td>
<td>Depletion of PIP2 in cytoplasmic vesicles by inositol phosphatases abolishes Ca2+ oscillations at fertilization. Defects/deficits in PIP2 and/or reduced amounts of oocytes vesicles containing PIP2 could contribute to OAD.</td>
<td>Yu et al. (2008); Yu et al. (2012)</td>
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<tr>
<td>InsP3R</td>
<td>InsP3 binds to InsP3R located on the endoplasmic reticulum membrane. InsP3R then opens an intrinsic channel and allows Ca2+ release from the ER.</td>
<td>Inhibition of InsP3Rs abolishes Ca2+ oscillations. Genetic mutations leading to abnormal protein folding or abnormal trafficking of InsP3Rs could result in OAD.</td>
<td>Cooney et al. (2010); Jellerette et al. (2000); Miyazaki et al. (1993); Ross et al. (2008); Wójcikiewicz et al. (1994)</td>
</tr>
<tr>
<td>PKC</td>
<td>PKC is activated by DAG and phosphorylates different substrates, including MARCKS. PKC is suggested to phosphorylate and activate other unidentified membrane channels.</td>
<td>Inhibition of PKC terminates sperm-induced Ca2+ oscillations and prevents Ca2+ influx that takes place after Ca2+ depletion in the ER. Genetic mutations leading to abnormal protein folding could contribute to OAD.</td>
<td>Gallicano et al. (1997); Gonzalez-Garcia et al. (2013); Halet et al. (2004); Luna et al. (2000); Tatone et al. (2003); Yu et al. (2008)</td>
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InsP3R: InsP3 receptor; PIP2: phosphatidylinositol 4,5-bisphosphate; PKC: protein kinase C; MARCKS: myristoylated alanine-rich C-kinase substrate; ER: endoplasmic reticulum.

PIP2 from sources other than the plasma membrane, and why PLCζ activity is oocyte-specific. While one could venture that the C2 domain is involved in targeting PLCζ from these vesicles, as happens with this domain in other PLC isoforms (Swann and Lai, 2013), the existence of this oocyte protein would also explain why C2 domains from PLCζ, but not those from other PLCs, are able to hydrolyse PIP2 from oocyte vesicles (Fig. 2).

Finally, the existence of such an oocyte receptor could explain some cases of OAD and ICSI failure, in which the cause is not thought to be a deficiency in sperm PLCζ. Therefore, more research is warranted to confirm this hypothesis and to address whether this unidentified protein could represent a prognostic marker with clinical and therapeutic implications.

**PIP2 and DAG**

Apart from suggestions regarding a putative PLCζ interactors in the oocyte, other oocyte factors that take part in the PLC pathway (such as PIP2, DAG, InsP3R, or Ca2+ channel proteins) could also be involved in OAD (Fig. 2 and Table II). Of these additional factors, one could suggest that PIP2 is a logical starting point as there is a considerable number of vesicles throughout the ooplasm containing PIP2 that are specifically targeted by PLCζ (Yu et al., 2012). As downstream events leading to embryogenesis depend on the availability of InsP3 and DAG, and these depend upon the availability of sufficient amounts of PIP2, it could be hypothesized that an abnormally low content of PIP2 in oocyte vesicles, or a reduced number of vesicles, could readily lead to OAD (Table II).

This suggestion is supported by previous studies in which depletion of PIP2 from cytoplasmic vesicles by inositol phosphatases led to the abolishment of Ca2+ oscillations following fertilization (Yu et al., 2008). However, comparative studies evaluating the number of vesicles in different oocytes have yet to be conducted.

Although there is agreement concerning PLCζ and its ability to hydrolyse PIP2, only a limited number of studies have focused on the role of DAG. In this regard, it appears that not only PIP2 from the plasma membrane is unaffected after injection of PLCζ, but also DAG generation in the plasma membrane is relatively minimal (Yu et al., 2008). Significant production of DAG in the ooplasm occurs when PKCζ1 (a PKC isoform) rather than PLCζ, is over-expressed and this is concomitant with a series of unexpected secondary high-frequency Ca2+ oscillations. These secondary Ca2+ oscillations can be mimicked in a variety of situations by the stimulation of PKC and can be prevented by PKC inhibition, so that overproduction of DAG in PKCζ1-injected oocytes can lead to PKC-mediated Ca2+ influx and subsequent overloading of Ca2+ stores (Yu et al., 2008).

While PIP2 has been proven to exist in vesicles distributed across the oocyte cytoplasm, it remains to be confirmed whether PLCζ-generated DAG remains in the membrane of such vesicles. In addition, transverse diffusion (or flip-flop) of DAG across the two leaflets of the lipid bilayer depends on the fluidity of the membrane bilayer, which in turn relies upon the composition and ratio of cholesterol/unsaturated glycerophospholipids in this membrane. This is important since the regulation of transbilayer movement of DAG may modify its binding to the C1 domain of PKC and this could, in turn, affect the downstream PKC-activated cascade (Ueda et al., 2014).

**The central role of InsP3R**

**Isoforms and regulation**

InsP3 interacts with its receptor (InsP3R), a ligand-gated channel, via two phosphate groups that bind to opposite sides of the InsP3-binding site (Fig. 2). This leads to a conformational change that opens the channel and allows Ca2+-release from internal stores, such as the ER (Bhanumathy et al., 2012). The final InsP3R structure arises from the assembly of four identical (homotetramer) or different subunits (heterotetramer), and this is reliant upon the cell type (Alzayady et al., 2013). Up to three different isoforms for this receptor (InsP3R1, InsP3R2, InsP3R3) have been described thus far (Ivanova et al., 2014).
Evidence for the role of the InsP₃R during oocyte activation and maturation

InsP₃R levels are correlated with variations in Ca²⁺ levels (retention of Ca²⁺ in the cytosol sensitizes the InsP₃R) and, after fertilization, high levels of InsP₃R inevitably lead to the down-regulation of InsP₃Rs (Wojcikiewicz et al., 1994; Parrington et al., 1998b; Jellerette et al., 2000; Ross et al., 2008; Cooney et al., 2010). Indeed, the degradation of InsP₃R depends upon uniform and sustained elevated levels of InsP₃, which results in a diminished periodicity of calcium oscillations (Miyazaki et al., 1993; Lee et al., 2010). Further evidence of the role of InsP₃ in InsP₃R down-regulation is that adenophosnin, a potent agonist for InsP₃R (Sato et al., 1998), down-regulates InsP₃R but does not trigger the activation of oocytes (Brind et al., 2000). In addition, down-regulation of InsP₃R may not completely prevent Ca²⁺ oscillations (Malcuit et al., 2005). Therefore, while Ca²⁺ oscillations are almost entirely mediated by InsP₃R, it remains to be determined whether all Ca²⁺ is released following InsP₃ interaction with InsP₃R, or whether other calcium stores are involved (Table II).

On the other hand, InsP₃R1 not only plays a relevant role for the activation of mammalian oocytes but also for their maturation and early embryo development. Indeed, the resumption of meiotic division in immature oocytes (i.e. at germinal vesicle stage) is also mediated by a phosphoinositide pathway involving InsP₃ present both in the ooplasm and nucleoplasm. In this context, it is worth mentioning that the nuclear membrane also presents InsP₃R1 and blocking these receptors leads to the inhibition of germinal vesicle breakdown (Pesty et al., 1998).

In addition, during oocyte maturation there is an increase in the sensitivity of InsP₃R1-mediated Ca²⁺ release, mediated by the phosphorylation of the Thr₁₅₅₈ residue in InsP₃R1 by polo-like kinase 1 (PLK1), that may ultimately affect the oocyte’s ability to be activated (Xu et al., 2003). The relevance of PLK1 is apparent from studies showing that PLK1-inactivation in mouse oocytes at the early stages of maturation significantly reduced InsP₃R1 phosphorylation (Vanderheiden et al., 2009). However, regulation of InsP₃R1 is a far more complex process since it does not only involve phosphorylation by PLK1 but also by mitogen-activated protein kinase/extracellular regulated kinase 2 (MAPK/ERK2) (Lee et al., 2006).

Finally, InsP₃R1 can be involved in the apoptosis pathway, since Caspase 3 is able to cleave InsP₃R1, thereby generating a truncated form of this receptor. This truncated InsP₃R1 consists of a C-terminal fragment of 95 kDa that comprises the complete channel domain. Interestingly, aged mouse oocytes present this truncated form, which prevents Ca²⁺ oscillations to be evoked and induces an apoptotic phenotype when it is injected into mouse oocytes. This explains OAD in aged oocytes, since they are diverted into an apoptosis pathway (Verbret et al., 2008).

InsP₃Rs are present in oocytes from mammalian and non-mammalian species, but their intracellular distribution can differ between species

The presence and relevance of InsP₃Rs for oocyte activation was first reported in Xenopus oocytes, where they were seen to organize into microscopic and macroscopic clusters (Mak and Foskett, 1997; Zhang et al., 2007). InsP₃R1 is present in the ER and perinuclear region of these immature oocytes, and relocates during oocyte maturation and following fertilization (Kume et al., 1993, 1997; Toranzo et al., 2014). In addition, InsP₃R3 is also present in the plasma membrane of Xenopus oocytes and for this reason is thought to be involved in Ca²⁺ entry from the extracellular space (Putney, 1997). However, this does not seem to be the case for mammalian oocytes, as discussed in the following section.

In pre-ovulatory and M-II mouse oocytes, InsP₃R1 is distributed across the cytosol (i.e. from cortex to perinucleus) and its expression significantly increases during oocyte maturation. This indicates that there is a propagating Ca²⁺ release from the cortex to the interior of the oocyte that is involved in oocyte maturation. In contrast, InsP₃R2 (aggregated in isolated islands in the cortex) and InsP₃R3 are also present but in lower amounts than InsP₃R1 (Fissore et al., 1999b).

In bovine species, the three isoforms are also expressed in M-II oocytes, but again InsP₃R1 is present in a larger quantity than the others. InsP₃R1-downregulation occurs following fertilization and pronuclei formation indicating that this isoform participates in oocyte activation (He et al., 1999). In pigs, localization and expression changes of InsP₃R1 during oocyte maturation are thought to be mediated by PLK1, and no differences in the distribution of InsP₃R1 were observed between in vivo and in vitro maturation. Furthermore, it is worth noting that while InsP₃R1 forms large clusters in the cortex of mouse oocytes, these structures are not observed in their pig counterparts (Sathanawong et al., 2015). In addition, it is worth noting that total expression levels and distribution of InsP₃R1 in the oocyte cytoplasm have been reported to be affected by vitrification-warming procedures in both mouse and pig oocytes (Kim et al., 2011; Hirose et al., 2013).

In humans, InsP₃R1 changes from a diffuse, irregular and granular distribution in immature oocytes (i.e. at the germinal vesicle stage) to a reticular and peripheral localization at the M-II stage. After fertilization, InsP₃R1 distribution changes again from a localization in the periphery and centre in zygotes and 2–4-cell embryos to a predominant pattern across the cytosol in 6–8-cell embryos. Apart from changes in the distribution of InsP₃R1, there is also an up-regulation of this receptor from immature to mature oocytes and from zygotes to 6–8-cell embryos (Goud et al., 1999).

PKC

The PKC family

Following the downstream cascade, another protein that plays a key role during oocyte activation and at the beginning of embryogenesis is PKC (Fig. 2). Protein kinases are involved in transduction pathways and phosphorylate serine (Ser), threonine (Thr) and tyrosine (Tyr) residues. Specifically, PKC is a ubiquitous Ser/Thr-protein kinase that is involved in the
phosphoinositide pathway and is activated by Ca\textsuperscript{2+} and DAG (Inoue et al., 1977; Nishizuka, 1992).

Three different sub-families have been described according to their structural and activation features: conventional PKCs (α, βI, βII and γ); non-classic, also known as novel, PKCs (δ, ε, η and θ); atypical PKCs (ζ and η/λ). While conventional PKCs are activated by DAG and require phosphatidylserine and Ca\textsuperscript{2+} as cofactors, non-classic PKCs are activated by DAG and phosphatidylserine but not by Ca\textsuperscript{2+}, and atypical PKCs only depend upon phosphatidylserine (i.e. are independent from DAG or Ca\textsuperscript{2+}) (reviewed by Kang et al., 2012). Some of these isoforms are present in all tissues, while others are tissue-specific.

All members of the conventional family (α, βI, βII and γ) present four conserved domains (C1–C4). The first domain (C1) presents cysteine-rich zinc finger-like motifs and the recognition sites for DAG, phosphatidylserine, and phorbol ester. The second conserved domain, known as C2, contains the binding site for Ca\textsuperscript{2+} and is rich in acidic residues. Finally, C3 and C4 domains are those that contain binding sites for ATP and substrates (Newton, 1995; Kanashiro and Khalil, 1998). Although it is well understood that conventional PKCs are activated by DAG and by Ca\textsuperscript{2+} released from the ER after InsP\textsubscript{3}-stimulation, the full list of substrates that PKC is able to phosphorylate has yet to be reported. In this regard, one could venture that substrates for PKC include unidentified membrane channels, since PKC is also involved in store-operated calcium influx (See below in Section about Ca\textsuperscript{2+} oscillations and homeostasis in the oocyte; Halet et al., 2004).

**PKC and the mammalian oocyte**

Different PKCs are known to be present in mammalian germinal vesicle and M-II arrested oocytes. Indeed, mouse and rat oocytes are known to exhibit conventional (PKC\textalpha, PKC\textbeta\textI and PKC\textgamma), non-classic (PKC\textdelta and PKC\textepsilon) and atypical isoforms (PKC\textzeta and PKC\texteta/lambda) (Jones, 1998; Pauken and Capco, 2000; Baluch et al., 2004).

One interesting issue relates to the distribution of conventional PKC isoforms. Prior to oocyte activation, PKC is uniformly distributed in the cytoplasm and PKC\textbeta\textI and PKC\textbeta\textII are both present in the cytoplasm and in the plasma membrane. Following sperm–oocyte fusion, PKC\textalpha translocates to the oocyte cortex. This translocation is considered a key feature of oocyte activation with kinetics similar to the Ca\textsuperscript{2+} oscillations induced by sperm. This dependency in Ca\textsuperscript{2+} oscillations is mediated by the C2 domain of PKC (Luria et al., 2000; Halet et al., 2004). Furthermore, while levels of PKC\textbeta\textI in the ooplasm decrease after oocyte activation, the distribution of PKC\textbeta\textII remains unaffected.

On the other hand, Chen et al. (2014) have suggested that PKC\textdelta and PKC\textepsilon are also involved in oocyte maturation. Indeed, these two proteins appear to mediate the response to FSH in cumulus oocyte complexes, which ultimately results in the meiotic resumption of germinal vesicle oocytes.

**The role of PKC\textalpha during oocyte activation**

The role of PKC\textalpha in oocyte activation has been confirmed by its involvement in the generation of the second polar body and the exocytosis of cortical granules (Gallicano et al., 1997; Ellyahu and Shalgi, 2002; Fig. 2). In fact, not only does PKC\textalpha activity increase after fertilization and return to baseline levels following Ca\textsuperscript{2+} oscillations (Gallicano et al., 1997; Tatone et al., 2003), but also inhibiting PKC\textalpha with Bisindolylmaleimide-I leads to OAD (Halet, 2004). Increased PKC\textalpha activity has been observed in the plasma membrane but also in the cytoplasm, the increase in the latter supporting the existence of vesicles containing PIP\textsubscript{2} and hydrolysed by PLC\textgreek{zeta} at fertilization (Gonzalez-Garcia et al., 2013) (Table II).

As mentioned previously, the extrusion of cortical granules is also mediated by PKC\textalpha (Talmor-Cohen et al., 2004). Upon fertilization, PKC\textalpha translocation to the plasma membrane allows phosphorylation of myristoylated alanine-rich C-kinase substrate (MARCKS) proteins, which are involved in the regulation of actin network and thus in the prevention of polyspermy (Ellyahu et al., 2005, 2006). Indeed, phosphorylation of MARCKS leads then to disassembly of the actin network at the oocyte cortex and allows cortical granules to reach the oolemma and to exocytose (Tsadon et al., 2008). This explains why it is relevant for PKC to be translocated to the membrane following sperm-membrane fusion as an event of oocyte activation.

It is worth noting that conventional PKCs have been suggested as chronometers, because they first translocate to the oolemma during oocyte activation where they mediate ‘store-operated calcium entry’ (SOCE) and phosphorylate MARCKS. Subsequently, PKCs are converted into their catalytic subunits (PKC-M) that travel to the ooplasm and phosphorylate cytoskeletal components and other substrates (Capco, 2001; Kalive et al., 2010).

It has yet to be reported whether any calcium pump involved in producing Ca\textsuperscript{2+} oscillations in mammalian oocytes is phosphorylated by PKCs. This could also explain the failure of oocytes to activate. This suggestion comes from studies conducted with Xenopus oocytes, in which voltage-gated calcium (Cav2.2) channels were found to be a phosphorylation substrate for PKCs. It is known that Thr\textsubscript{422}, Ser\textsubscript{1757} and Ser\textsubscript{2132} residues within Cav2.2 channels are target sites for PKC\textalpha (Rajagopal et al., 2014).

Finally, some reports have questioned the role of PKC\textalpha during oocyte activation, since CaMKII is able to trigger meiotic resumption and early development (Madgwick et al., 2005; Knott et al., 2006), while others have indicated that PKC activity in the plasma membrane upon fertilization is marginal (Yu et al., 2008). Therefore, more research is required to address whether abnormalities in PKC may underlie OAD.

**PKCs, oocyte maturation and early embryo development**

The role of PKC is also important in meiotic resumption of the oocyte in a process that involves zinc, as free zinc levels in the cytoplasm significantly increase during oocyte maturation and the depletion of zinc using a blocking agent reduces levels of both phosphorylated MAPK and PKC. In contrast, phosphorylation of PKC substrates and MAPK increase when oocytes are treated with a zinc agonist (Zhao et al., 2014b). Moreover, in a study that evaluated the effect of age, germinal vesicle transfer and modified nucleoplasmic ratio on PKC\textalpha distribution, it was observed that while PKC\textalpha presented similar localization patterns within the cytoplasm of M-II oocytes and early embryos, it mainly resided in the nucleus after pronuclei formation and in 2-cell embryos (Cui et al., 2012).

As previously mentioned, non-classic and atypical PKCs have also been reported to play a role during oocyte maturation, fertilization and cell differentiation during preimplantation development (Carracedo et al., 2013; Saiz et al., 2013). Indeed, non-classic PKC\textdelta and atypical PKC\textzeta reside in the meiotic spindle and are suggested to play a role during meiosis (Tatone et al., 2003; Viveiros et al., 2003; Baluch et al., 2004). In humans, PKC\textdelta has been demonstrated to modulate the...
epithelial mesenchymal transition from embryonic stem cells to cells expressing markers of parietal endoderm, an extraembryonic lineage formed before the formation of primitive ectoderm (Feng et al., 2012). Finally, Ezrin, an important protein involved in the compaction of 8-cell embryos, possesses a Thr567 residue that is phosphorylated by an atypical PKC, which then leads to embryo compaction (Liu et al., 2013a).

**Ca^{2+} oscillations and homeostasis in the oocyte**

Calcium is a ubiquitous secondary messenger that plays a fundamental role in many reproductive processes, such as sperm capacitation and acrosome reaction (Costello et al., 2009; Alasmari et al., 2013; Yeste et al., 2015). We have already mentioned how important Ca^{2+} oscillations are for the events that occur immediately after fertilization (i.e., oocyte activation and early embryo development), and the key role of SOAF in evoking such oscillations. Indeed, these oscillations have been described to present complex temporal and spatial properties, beginning soon after gamete fusion and persisting beyond the completion of meiosis (Parrington et al., 1998b; Saunders et al., 2002; Swann et al., 2004, 2006; Kouchi et al., 2005; Sone et al., 2005; Ito et al., 2011). Furthermore, the Ca^{2+} balance within and outside the oocyte is critically important, since extracellular Ca^{2+} levels in the ER lumen decrease, STIM1 translocates to the plasma membrane, and influx from the extracellular space (Putney, 1986, 2011; Smyth et al., 2010). The major components of this system are stromal interaction molecule-1 (STIM1), Ca^{2+} release-activated Ca^{2+} channel protein 1 (ORAI1), sarco/endoplasmic Reticulum Ca^{2+} ATPase (SERCA) and other unidentified membrane channels (Table III and Fig. 3). As InsP_{3} is known to intervene in the release of Ca^{2+} from ER, it is reasonable to suggest that abnormalities in these proteins could affect Ca^{2+} release and thus the oocyte's ability to be activated.

The relevance of SOCE, known to play vital functions in other cell types, has also been demonstrated in mammalian oocytes (Martínez-Romero et al., 2012). Two components of this system, STIM1 and ORAI1, are important during oocyte maturation, when there is a progressive Ca^{2+} influx to the oocyte and a subsequent Ca^{2+}-increase in the oocyte internal stores.

The pattern of Ca^{2+} release in artificially activated oocytes differs from that evoked by PLC_{6} following normal fertilization (Yanagida et al., 2005; Kline and Kline, 1992b). However, more research is still required to address this point.

### Table III  Potential oocyte factors with implications for OAD (II): elements of the store-operated calcium-entry system.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Description</th>
<th>Possible contribution to OAD</th>
<th>References</th>
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<tbody>
<tr>
<td>ORAI1</td>
<td>ORAI1 is a Ca^{2+} channel located in the plasma membrane. Upon stimulation by STIM1, ORAI1 channels open and allow Ca^{2+} influx.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERCA</td>
<td>SERCA is a pump located in the ER membrane that pumps Ca^{2+} into the ER lumen. Therefore, the ER can be refilled with cytosolic Ca^{2+}.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unidentified membrane channels</td>
<td>Since inhibition of SOCE proteins does not completely abrogate Ca^{2+} oscillations, the existence of other, unidentified Ca^{2+} membrane channels is suggested. However, more research is still required to address this point.</td>
<td></td>
<td>Miyazaki (1988)</td>
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ORAI: Ca^{2+} release-activated Ca^{2+} channel protein 1; SERCA: sarco/endoplasmic Reticulum Ca^{2+} ATPase; STIM1: stromal interaction molecule-1; SOCE: store-operated calcium-entry system.
mainly the ER (Cheon et al., 2013). Such an increase has been related to the key role of this second messenger during oocyte activation. STIM1 is a single-pass, type-I transmembrane protein that activates Ca\textsuperscript{2+} entries into stores when luminal levels of this second messenger are low (Roos et al., 2005; Zhang et al., 2005). STIM1 is present in the ER membrane but can also be found in the plasma membrane (Soboloff et al., 2012). The primary sequence of this protein contains 685 amino acids and the cytosolic region presents two coiled-coil domains that are rich in proline/serine and lysine, respectively. The luminal region contains a signal peptide for ER, an EF-hand domain, and a sterile alpha motif domain (Williams et al., 2002; Stathopulos et al., 2008). The EF-hand and sterile alpha motif domains act as a sensor of Ca\textsuperscript{2+} levels, since when Ca\textsuperscript{2+} levels in the ER decrease, the sterile alpha motif, which is adjacent to the EF-hand domain, mediates the oligomerization of STIM1 (Liou et al., 2007). Therefore, these two regions are crucial for a proper functioning of the SOCE mechanism, and mutations in the EF-hands, or sterile alpha motif domains, may lead to active Ca\textsuperscript{2+} influx regardless of Ca\textsuperscript{2+} levels within the store. Considering that Ca\textsuperscript{2+} plays a crucial role in downstream events triggered by the SOAF and release from the ER, it could be the case that alternations/deficiencies in this protein ultimately affects the regulation of normal Ca\textsuperscript{2+} oscillations, as the folded/unfolded state of the Ca\textsuperscript{2+} sensor in STIM1 is vital for SOCE regulation (Stathopulos et al., 2008).

However, more research is warranted in this respect since such deficiencies could impact upon other cell functions across the entire body. STIM1 function is related to ORAI1. In a suggested general picture of the process (Fig. 3), the SOAF leads to the hydrolysis of PIP\textsubscript{2} into InsP\textsubscript{3} and DAG, and InsP\textsubscript{3} interacts with its receptor located on the ER membrane. Ca\textsuperscript{2+} is then released from the ER, which concomitantly reduces Ca\textsuperscript{2+} levels in the ER lumen. This reduction in Ca\textsuperscript{2+} is detected by the EF-hand and sterile alpha motif domains of STIM1, and this leads this protein to cluster and relocate to the plasma membrane. STIM1 then interacts with the cytoplasmic region of the ORAI1 channel, a structural component of Ca\textsuperscript{2+} release-activated ion channels, and this leads to a conformation change near the external entrance to the pore that opens the channel and allows Ca\textsuperscript{2+} influx (Feske et al., 2006; Barr et al., 2009; Putney, 2009; Gudlur et al., 2014). Interestingly, STIM1 presents a Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} activation domain that consists of a highly conserved region of 107 amino acids that binds directly to the N and C termini of ORAI1. Therefore, the direct protein–protein interaction between STIM1 and ORAI1 leads to set junctions between the ER and plasma membrane (Park et al., 2009). Therefore, deficiencies in ORAI1 could lead to the oocyte being unable to flux Ca\textsuperscript{2+} properly, and result in the inability of oocytes to elicit proper Ca\textsuperscript{2+} oscillations (Lee et al., 2012; Wang et al., 2012).
Sarco/endoplasmic reticulum and plasma membrane Ca\(^{2+}\)-ATPases Sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA) are P-type ion pumps located in the ER membrane that pump Ca\(^{2+}\) from the cytosol to the lumen of the ER (Ullah et al., 2007; Fig. 3). The genes encoding these proteins (SERCA1, SERCA2 and SERCA3) are located on three different chromosomes and up to 14 SERCA-transcripts, with alphanumerical identification, are generated by alternative splicing. This depends upon cell type and developmental stage (Periasamy and Kalyanasundaram, 2007). In addition, some of these isoforms have been reported to be species-specific, such as SERCA2c, SERCA3d, SERCA3e and SERCA3f, which are only present in humans (Dally et al., 2010). During Ca\(^{2+}\) oscillations, the ER needs to be refilled with calcium after the luminal levels are depleted. In this context, SERCA pumps Ca\(^{2+}\) to the ER in order to generate another Ca\(^{2+}\) oscillation that will be released upon interaction of InsP\(_3\) with its receptor (Wang and Machaty, 2013).

SERCA2b is an isoform present in mammalian oocytes that, when inhibited with thapsigargin during oocyte maturation, prevents the increase of Ca\(^{2+}\) within the ER (Kline and Kline, 1992b). A two-step model has been proposed to explain the replenishment of Ca\(^{2+}\) in the ER, which consists of a rapid Ca\(^{2+}\) uptake from the cytosol by SERCA proteins and a gradual phase that relies upon Ca\(^{2+}\) influx from the extracellular milieu (Wakai et al., 2013).

SERCA2b is redistributed in sub-cortical areas located close to the InsP\(_3\)Rs of M-II oocytes and is organized into clusters when the oocyte reaches the M-II stage (Wakai et al., 2013). While SERCA2b clusters are present within 3 h post-fertilization, they disappear as the pronuclear stage is reached. Since this matches with the actual duration of Ca\(^{2+}\) oscillations in mice (Jones et al., 1995), it confirms the relevance of this protein in the events that take place after sperm–oocyte fusion (Wakai et al., 2013) (Table III).

Other vital proteins for Ca\(^{2+}\) oscillations include plasma membrane Ca\(^{2+}\)-ATPases (PMCA), which pump Ca\(^{2+}\) from the cytosol to the extracellular milieu. Therefore, inappropriate functioning of SERCA and PMCA pumps has been suggested to disrupt Ca\(^{2+}\) oscillations and lead to an increase in cytosolic Ca\(^{2+}\) levels (Wakai et al., 2013). However, while deficiencies in PMCA could feasibly underlie OAD, more research is still required to investigate their presence and function in mammalian oocytes.

### Other oocyte proteins that could underlie oocyte activation failure

#### CaMKII and MAPK

Ca\(^{2+}\) oscillations allow the resumption of meiosis as they switch on calcium/calmodulin-dependent protein kinase II (CaMKII) (Dupont, 1998; Von Stetina and Orr-Weaver, 2011; Fig. 2). Indeed, upon activation, CaMKII phosphorylates the oocyte-specific protein Emi2 (Madgwick et al., 2005; Ducibella et al., 2006; Knott et al., 2006). Emi2 allows the oocyte to remain arrested at M-II as it prevents the loss of M phase/maturation-promoting factor (MPF), a heterodimer of cyclin-dependent kinase 1 (CDK1) and CCNB1, by blocking CCNB1-degradation (Jones, 2007). When Emi2 is phosphorylated by CaMKII and thus degraded, MPF is also degraded and the oocyte is able to alleviate its M-II arrest (Fig. 2). On the other hand, high cytoplasmic levels of Ca\(^{2+}\), via CaM and CaMKII, inhibit InsP\(_3\)Rs. Since this is followed by a reduction in the release of Ca\(^{2+}\) from internal stores and thus a reduction in the concentration of Ca\(^{2+}\) in the ooplasm, InsP\(_3\)R inhibition terminates and Ca\(^{2+}\) release can be renewed from the stores (Matifat et al., 2001). Therefore, as CaMKII is a Ca\(^{2+}\)-dependent protein kinase, lack of appropriate Ca\(^{2+}\) homeostasis can prevent the protein from functioning, and thus impact upon oocyte activation (Table IV).

On the other hand, following resumption and termination of meiosis-II, the pronuclei are formed in a process that is also dependent on Ca\(^{2+}\) levels and involves the MAPK pathway (Ducibella and Fissore, 2008; Fig. 2). Thus, deficiencies in this transduction pathway could prevent the oocyte from undergoing embryogenesis.

#### Other phospholipases

In the debate to elucidate which sperm protein is the actual SOAF, it is fundamentally clear that any protein candidate needs to be intimately involved in Ca\(^{2+}\) metabolism and homeostasis. Different PLCs (β, γ,

<table>
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<tr>
<td>CaMKII</td>
<td>CaMKII is one of the proteins activated by Ca(^{2+}). This protein plays a key role in the activation of oocytes, since it initiates a cascade that leads to alleviation of meiotic arrest.</td>
<td>Genetic mutations in these proteins resulting in loss of function may prevent events of oocyte activation taking place, even when Ca(^{2+}) oscillations are generated successfully.</td>
<td>Ducibella et al. (2006)</td>
</tr>
<tr>
<td>MAPK</td>
<td>Inactivation of MAPK by Ca(^{2+}) oscillations allows pronuclei formation.</td>
<td></td>
<td>Dupont (1998)</td>
</tr>
<tr>
<td>Oocyte phospholipases</td>
<td>PLC(<em>{β}), γ and δ are found in the ooplasm and may have a role in generating Ca(^{2+}) oscillations. In addition, PAWP has been suggested to interact with PLC(</em>{γ}).</td>
<td>Reduced PLC(<em>{β})I in mouse eggs is linked to Ca(^{2+}) oscillations of lower amplitude, so loss/ malfunction of oocyte PLC(</em>{β})I could lead to OAD. If PAWP is confirmed to interact with PLC(<em>{γ}), and PAWP plays a key role for oocyte fertilization, a malfunction of oocyte PLC(</em>{γ}) could lead to OAD and/or fertilization failure.</td>
<td>Aarabi et al. (2014a)</td>
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</table>

δ), but not ζ, are found in the ooplasm of mammalian oocytes (Saunders et al., 2002). These PLC isozymes appear to play a role in Ca2⁺ oscillations, since Igarashi et al. (2007) found that when levels of PLCδ1 in mouse oocytes were reduced, Ca2⁺ oscillations were of lower amplitude (Table IV). In amphibians, it was reported that Ca2⁺ can activate a cytosolic phospholipase A2 (cPLA2), which releases arachidonic acid from phospholipids of the ER as well as from the nuclear envelope, in a step that would make this protein able to participate in oocyte activation (Ajmat et al., 2013). For this reason, we could also suggest that alterations/deficiencies in other phospholipases within the oocyte could also lead to OAD.

**CDK1 and CHERP**

In ascidians, the sequestration of PLCζ does not stop Ca2⁺ spikes. Instead, it seems that it is the blockage of CDK1 that causes termination of Ca2⁺-induced spikes. Following these results, it has been suggested that CDK1, an oocyte protein, promotes InsP3 generation in the presence of PLCζ, thereby indicating that CDK1 is linked to phosphoinositide pathway (Levasseur et al., 2007, Fig. 2).

Another ubiquitous protein that is known to be involved in Ca2⁺ trafficking in all cell types is Ca2⁺ homeostasis endoplasmic reticulum protein (CHERP). This protein has been suggested to regulate Ca2⁺ channels in the ER membrane (i.e. InsP3Rs and ryanodine receptors), via regulating the function of the U2 small nuclear RNA splicingosomal complex in the nucleus rather than acting as a cytoplasmic regulator (Lin-Moshier et al., 2013). In Xenopus oocytes, CHERP is present in the nucleus rather than in the ER, so its functional role in the cytoplasm needs to be demonstrated (Lin-Moshier et al., 2013). Studies investigating the presence and putative role of CHERP have yet to be conducted in mammalian oocytes.

**Oocyte mitochondria and Ca2⁺ oscillations**

While fundamentally involved in oxidative phosphorylation, mitochondria also represent important internal stores of Ca2⁺ (Dumont et al., 2004; Fig. 3). In this regard, it has been suggested that oocytes with fewer mitochondria and/or inappropriate mitochondrial function, and thus reduced levels of ATP production, could fail to exhibit normal Ca2⁺ oscillatory patterns and therefore influence activation ability. In addition, not only the absence of an appropriate balance between Ca2⁺ release and sequestration has coincident effects upon levels of mitochondrial ATP (Van Blerkom, 2011), but also excessive Ca2⁺ oscillatory activity may result in the oocyte entering an apoptotic mitochondria-caspase mediated pathway, as demonstrated earlier in rat oocytes cultured in vitro (Tripathi and Chaque, 2012). These data have led to the belief that mitochondrial abnormalities in the oocyte may underlie some cases of oocyte-related OAD (Van Blerkom, 2011).

**Lessons from the in vitro maturation of human oocytes**

In vitro maturation (IVM) of human oocytes is a promising technique for fertility preservation. Increasing success rates for this technique may benefit, amongst others, young women suffering from cancer that cannot delay chemotherapy and/or cannot be offered ovarian hyperstimulation (Chian et al., 2013). Although success rates for IVM in human oocytes have been increasing over recent times, there are still some issues that need to be addressed. While it is understood that oocyte maturation consists of complex changes that involve both the nucleus and cytoplasm, it is also considered that alterations in organelles and the cytoskeleton that occur during IVM are all possible causes for OAD, fertilization failure and reduced developmental embryo development (Mao et al., 2014; Neri et al., 2014). Supporting this, Ca2⁺ oscillations observed in IVM-fertilized human oocytes are of lower frequency and shorter duration than those observed in oocytes matured in vivo (Nikiforaki et al., 2014). In addition, Liu et al. (2013b) showed that vitrified-warmed and subsequently in vitro matured human oocytes presented higher ICSI-fertilization success when AOA was used. These findings match the idea that the cytoplasmic changes that take place during oocyte maturation affect the machinery that regulates Ca2⁺ homeostasis and are vital for the sperm-triggered events following sperm-oocyte fusion.

The asynchrony between nuclear and cytoplasmic maturation during oocyte maturation, either in vivo or in vitro, can also account for the inability of the ooplasm to decondense the sperm chromatin (Eppig et al., 1994). In this context, the dialogue between the oocyte and the cumulus oophorus plays a key role during oocyte maturation, and seems to be responsible for allowing the oocyte cytoplasm to acquire the developmental competence required for fertilization success (Neri et al., 2014). While this hypothesis matches other studies involving human oocytes undergoing IVM (Hyun et al., 2007), more research into the IVM of human oocytes could also help to identify cases of OAD that are not due to a sperm factor deficiency.

In general, however, fertilization, implantation and childbirth rates with IVM are still lower than those achieved with conventional IVF (Son et al., 2008), and multinucleation rates following ICSI are higher in IVM than in in vivo-matured MII-oocytes (De Vincentiis et al., 2013). A number of factors might lead to lower implantation and childbirth rates with IVM, including non-synchronization of oocyte nuclear and cytoplasmic maturation, culture conditions, and timing of insemination. In particular, non-synchronization of oocyte nuclear and cytoplasmic maturation was considered to be a major reason for poor developmental competence of IVM oocytes (Combelles et al., 2002 and Moor et al., 1998). Chian et al. (2000) reported that HCG administration in vivo can hasten the nuclear maturation process, whereas its effect on cytoplasmic maturation is unclear. In addition, one of the important factors regulating the number and quality of oocytes maturing in vitro are the culture conditions and timing of insemination used for IVM.

**AOA**

**Mechanical, electrical and chemical activation**

AOA is a suitable technique for those couples that present a compromised fertilization rate below 30% following conventional ICSI (Heindryckx et al., 2005, 2008; Montag et al., 2012). This method involves the use of various mechanical, electric or chemical stimuli to help alleviate oocytes from MII-arrest (Alberio et al., 2001; Nasr-Esfahani et al., 2010; Vanden Meerschaut et al., 2014a). Not only has AOA been suggested to be effective in cases of sperm-related fertilization deficiency (Heindryckx et al., 2005), but also in some cases in which an oocyte cytosolic factor is suspected to underlie OAD (Vanden Meerschaut et al., 2012).
Mechanical activation is a modified ICSI procedure which involves vigorous cytoplasmic aspiration prior to injecting the sperm. This practice increases Ca\(^{2+}\) levels at the time of injection and helps the oocyte to activate (Ebenet et al., 2004). Electrical activation works by opening pores in the plasma membrane, thereby allowing Ca\(^{2+}\) influx from the surrounding media, ultimately resulting in an elevation of Ca\(^{2+}\) levels in the ooplasm. While these two approaches have been reported to be successful (Ebenet et al., 2004; Yanagida et al., 2008; Egashira et al., 2009), their success rates are lower than those obtained with chemical activation methods (Baltaci et al., 2010).

Chemical activation is the most commonly used procedure and consists of exposing oocytes to chemical agents that lead to an increase in intracellular Ca\(^{2+}\) levels in the oocyte. However, while some agents cause a single, prolonged Ca\(^{2+}\) transient in the oocyte, others cause multiple oscillations. Activating agents that cause a single Ca\(^{2+}\) transient include calcium ionophores, such as A23187 (also known as calcimycin) and ionomycin, and protein synthesis and kinase inhibitors, such as puromycin and 6-dimethylaminopurine, respectively (Tesarik et al., 2000; Yaman et al., 2000; Nakagawa et al., 2001; Murase et al., 2004; Lu et al., 2006; Heindryckx et al., 2008, 2011; Nasr-Esfahani et al., 2008; Terada et al., 2009). Within this category, calcimycin and ionomycin are the most used clinically. Their mechanism of action consists of increasing the permeability of the plasma membrane to Ca\(^{2+}\), thereby allowing extracellular Ca\(^{2+}\) to flow into the oocyte. The first case of AOA using calcimycin was reported in 1997 (Rybochkin et al., 1997), and recent data show fertilization rates from 25 to 48\%, with live birth rates of around 28\% (Ebenet et al., 2015). In addition, calcimycin was recently reported as being effective for the artificial activation of in vitro matured oocytes (Kim et al., 2015).

Chemical activators that cause multiple transients include strontium chloride (SrCl\(_2\)) (Kline and Kline, 1992a; Kishikawa et al., 1999; Kim et al., 2014), phorbol esters (Cuthbertson and Cobbold, 1985), thimerosal (Fissore et al., 1995) and anhydrous alcohol (Liu et al., 2013b). While SrCl\(_2\) is the most used, and has been reported to be effective in mice, its use in humans is still under debate (Yanagida et al., 2006; Kyono et al., 2008; Chen et al., 2010; Vanden Meerschaut et al., 2014a). Recently, Kim et al. (2014) showed that AOA with SrCl\(_2\) may be a suitable strategy for those couples in which calcimycin is not effective enough. The mechanism by which SrCl\(_2\) induces Ca\(^{2+}\) oscillations is not fully understood, but appears to lead to Ca\(^{2+}\) release from the ER.

### Safety of AOA for child health

Although AOA has been proven as an efficient strategy with which to overcome some cases of TFF, OAD may result from multi-factorial causes, as discussed above. This should be taken into account when artificial activators are used, since using artificial activators that increase intracellular Ca\(^{2+}\) levels circumvents the normal oocyte machinery and Ca\(^{2+}\) transients are not regulated by the appropriate endogenous mechanisms. In addition, the use of AOA could also underlie some effects on gene expression and epigenetic regulation (Ozil et al., 2006; Ciapa and Arnoult, 2011). At present, a major concern over the use of AOA is that we know very little about downstream events in the oocyte and early embryo. For this reason, the safety of artificial activators has been the subject of much on-going debate (Nasr-Esfahani et al., 2010; Vanden Meerschaut et al., 2014a).

Although the number of live births registered after AOA still remains low, the use of calcium ionophores has been demonstrated to be safe and has not been linked with any deleterious effects in terms of child health (Heindryckx et al., 2005; Ebenet et al., 2012; Montag et al., 2012; Vanden Meerschaut et al., 2014b). While one study reported a baby born after calcimycin AOA with a major malformation (anal atresia) (Ebenet et al., 2015), it is entirely possible that this resulted from the use of ART, since the risk of congenital malformations is known to be increased following conventional IVF and ICSI (Källén et al., 2010; Kanasugi et al., 2013).

The first study investigating the subsequent health status of children conceived after combining AOA with ionomycin and ICSI has been published only very recently (Deemeh et al., 2014). Using a significant sample size (79 children born following AOA-ICSI and 89 born by ICSI), this study demonstrated the safety of AOA with ionomycin, since no significant differences between AOA-ICSI and conventional ICSI were found in terms of intrauterine fetal death, preterm delivery, birthweight, growth rate, hospitalization in neonatal intensive care units, abnormal behaviour according to age, and the physical and mental health of children born. In addition, Deemeh et al. (2014) also showed that AOA did not cause chromosomal abnormalities. This is highly relevant for safety because AOA takes place during meiotic spindle orientation and meiosis completion (Alberio et al., 2001). It is worth noting that the study by Deemeh et al. (2014) was conducted in couples that underwent ICSI with severe teratozoospermia or sperm collected by testicular extraction. This population is different from couples with previous failed fertilization cycles and, since the study by Deemeh et al. (2014) mainly focuses upon sperm, it does not address whether a problem in the relevant oocyte machinery has any impact on the health status of children born. In the case of SrCl\(_2\), data are less consistent. Indeed, while data in mice show that this treatment reduces the birthweight of female pups (Vanden Meerschaut et al., 2013), studies in humans have reported that it has no impact on the physical and mental development of children from birth to 60 months (Yanagida et al., 2006; Kyono et al., 2008; Kim et al., 2014).

### The use of AOA as a routine practice

Although the data reported so far are encouraging, AOA is yet to be considered an established treatment (Vanden Meerschaut et al., 2014a; Sfontouris et al., 2015). Indeed, while studies have reported no adverse effects, it must be noted that sample sizes are still too low and that data on the follow-up of children born following AOA remain scarce. Since the oocyte machinery governing Ca\(^{2+}\) homeostasis is bypassed by AOA, one should be prudent when assessing potential treatments given in this scenario. Indeed, there is still a lack of knowledge about the exact mechanisms of action triggered by AOA agents. While the impact of amplitude and frequency of artificially-induced Ca\(^{2+}\) oscillations on embryo development and gene expression has been reported (Ozil et al., 2006), the effects of calcium ionophores upon gene expression have yet to be studied. In addition, although the risk of abortion after conventional ICSI is higher than in natural conception (Deemeh et al., 2014), and there is an increased risk for some syndromes that has been associated with imprinting errors (Källén et al., 2010), future research should address whether the use of AOA increases these risks. Nevertheless, until a pure and active form of recombinant human PLC\(_{\gamma}\), or other SOAF protein(s), progresses through clinical trials and becomes commercially available, AOA remains the only treatment option for patients diagnosed with OAD.
Rescue of OAD with recombinant PLCζ

As mentioned above, Ca^{2+} oscillations generated by IVF and ICSI are known to differ from those generated when artificial oocyte activators are used and there are thus concerns about their potential side-effects (Swann and Yu, 2008; Yanagida et al., 2008). Related to this, it is worth noting that differences between mammalian and non-mammalian species exist in the specific patterns of Ca^{2+} oscillations elicited by PLCζ (Kashir et al., 2013a). Accordingly, injection of a non-mammalian PLCζ into a mouse oocyte evokes a single transient increase in Ca^{2+} levels and generates excessive DAG, which in turn leads to elevated Ca^{2+} entry (Yu et al., 2008). This impairs oocyte development, and at the same time suggests that an abnormal pattern of Ca^{2+} oscillations, such as that caused by chemical activators or PLCζ from a non-mammalian source, can be detrimental for embryogenesis. In this context, the production of recombinant human PLCζ protein, which should release Ca^{2+} within the oocyte in a more efficient and controlled manner than artificial activators, clearly represents a powerful therapeutic agent for OAD in those cases in which sperm exhibit PLCζ deficiency (Kashir et al., 2010; Nomikos et al., 2012). Strikingly, Yoon et al. (2008) were the first to demonstrate that cRNA encoding PLCζ could rescue fertility in an infertile patient whose sperm were both devoid of PLCζ and unable to induce Ca^{2+} oscillations following ICSI. Kashir et al. (2011) later published the synthesis of the first recombinant human PLCζ protein. When injected into mouse and human oocytes, this recombinant protein not only evoked Ca^{2+} oscillations that closely resembled those initiated by the sperm after fertilization but also led to cleavage (Kashir et al., 2011; Yoon et al., 2012). Moreover, the ability of recombinant human PLCζ to initiate development in mice embryos was lower than in human counterparts, thereby demonstrating the specificity of this recombinant form (Nomikos et al., 2013b).

Conclusions

While it is clear that sperm deficiencies can underlie a significant proportion of OAD cases, an intense debate has arisen concerning the true identity of the SOAF, with PLCζ and PAWP being the two key candidates (Aarabi et al., 2015; Amdani et al., 2015; Nomikos et al., 2015b; Vainos and Gerton, 2015; Table I). However, far less attention has been paid to the potential role of oocyte deficiencies in cases of OAD. The true relevance of oocyte-borne factors in OAD is clearly evident since total levels of TFF following ICSI (Yelumalai et al., 2015). Whether a specific oocyte receptor for PLCζ or PAWP exists within mammalian oocytes is something that still needs to be proven. Nevertheless, it is clear that apart from this putative receptor (or receptors), there are several other key oocyte proteins that are involved in the cascade triggered by the SOAF following sperm–oocyte fusion. In this regard, it would be very beneficial to identify which oocyte protein/s may also contribute to OAD, as this may generate new targets for clinical diagnostic assays or therapeutic intervention. In addition, Ca^{2+} trafficking is likely to involve many other, as yet unidentified, proteins. It follows then that deficiencies of oocyte activation could also be related to membrane channels that have yet to be identified. Genetic deficiencies and/or an aberrant expression of such proteins could prevent activation of the oocyte upon fusion with the sperm membrane.

Given the clear imbalance in ongoing studies pertaining to the relative role of sperm and oocyte proteins in OAD, it is clear that future research programmes should turn their attention to the oocyte. However, studying human oocytes presents particular difficulties, both from an ethical and practical point of view. For example, it is far more difficult to study a sufficient number of human oocytes in order to achieve statistical power than it is to study a sufficient number of sperm. In addition, while most existing studies utilize oocytes from animal models, it is already clear that the mechanism of PLCζ action may differ between species, and that this problem may also exist for other SOAF candidates.

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Authors’ roles

M.Y. wrote the manuscript. C.J., S.N.A., S.P. and K.C. revised the manuscript and approved the final version.

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Conflict of interest

The authors declare that there is no conflict of interest in regards to this manuscript.

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